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Cocoa Butter Injections, but not Sealed or Perforated Silastic Implants, of Corticosterone can be used to Chronically Elevate Corticosterone in Free-Living Painted Turtles (*Chrysemys picta*)

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Abstract.—**Chronic stress can result in an elevation of circulating levels of glucocorticoid (GC) hormones in vertebrates, which may affect their fitness. To isolate the effect of GCs on stressed organisms, one approach consists of manipulating circulating levels of GCs. We investigated the usefulness of two corticosterone (CORT) administration methods, Silastic® implants and cocoa butter injections, in chronically elevating circulating CORT levels in Painted Turtles (*Chrysemys picta*). First, free-living turtles received subcutaneous Silastic implants for 2 mo. We observed no significant difference in baseline CORT levels between two doses of CORT and sham-treated turtles. Then, captive turtles received a subcutaneous Silastic implant for 28 d. We observed no effect on baseline CORT levels, hormonal stress response, or body mass, suggesting that sealed and perforated Silastic implants of CORT may not be an effective way to elevate CORT in Painted Turtles. Second, we tested injections of CORT-laden cocoa butter for the first time in an ectothermic tetrapod. Free-living turtles received an epicoelomic injection of liquid cocoa butter mixed with CORT and were recaptured in the field over 2 mo. Despite large interindividual variation, we found that this injection approach generally kept circulating CORT levels elevated for up to 3 wk. Achieved CORT concentrations were probably physiologically and ecologically relevant for the species, although concentrations possibly remained elevated longer than would be the case in wild animals. Cocoa butter injections, but not sealed or perforated Silastic implants, can be used in Painted Turtles to chronically elevate CORT. Further, this represents a promising method for other temperate ectotherms such as amphibians and reptiles.**

When vertebrates face a stressful stimulus, they release hormones that help them cope with and survive the adverse conditions. One key aspect of the vertebrate stress response is the activation of the hypothalamic-pituitary-adrenal (HPA) axis, culminating in glucocorticoid (GC; corticosterone, CORT, in reptiles) release several minutes after initiation of a stressful stimulus (Sapolsky et al., 2000). The GCs then induce a variety of behavioral and physiological effects such as mediating the cardiovascular stress response, increasing circulating glucose concentrations and feeding behavior, and suppressing immunological and inflammatory responses as well as reproductive behavior (reviewed in Sapolsky et al., 2000). Collectively, these effects are believed to promote survival of the organism in the short term. Although the effects of an acute stressor generally fade within hours of the initial stimulus, sustained elevated levels of GCs can have negative physiological effects in the long term (Sapolsky et al., 2000), notably on growth, immunity, and reproduction (Guillette et al., 1995; Norris, 2007). Although subject to some debate, the term chronic stress can be defined as multiple, frequent exposure or long-term constant exposure (or both) to stressors (Cyr and Romero, 2009). Prolonged inclement weather and food deprivation, social interactions, habitat disturbance, and human activities are some examples of chronic stressors. Until recently, another definition was long-term allostatic overload, during which GC levels remain above baseline (i.e., within the range typically associated with acute stress, with type I and II GC receptors bound; Bonier et al., 2009). Boonstra (2013) argued that a stressor should be defined as acute or chronic not based on the duration of the stressor itself, but rather on the duration of its consequences on the physiology of the animal. Knowledge of patterns of GC secretion in wild animals is limited but expanding. A recent review in wild animals, however, revealed that chronically stressed animals do not necessarily exhibit a consistent, predictable, endocrine response to chronic stress; the fact that the response changes at all is more important than the direction of the changes (Dickens and Romero, 2013). Ultimately, chronic stressors can impact negatively the overall fitness of organisms and, eventually, their population dynamics (Bonier et al., 2009; Crespi et al., 2013).

In this context, a possible approach to determine the consequences of elevated GCs on an organism—for instance on stress responses, immune function, reproduction, or survival—consists of manipulating circulating levels of GC hormones. Although GC implantation does not represent a perfect mimic of stress, it provides an effective tool to examine a key constituent of the stress response, namely the rise in circulating GCs (Denardo and Licht, 1993). The direct manipulation of GCs eliminates the perceptual component of the stress response, reducing the complexity of stress-related questions (Denardo and Sinervo, 1994a,b). This allows the effect of stress on various fitness indicators, immunity, or reproductive success to be studied independently of any confounding effect of the actual stressor (e.g., habituation) or other related factors (e.g., parasite density or habitat variations).

Several methods have been used to administer GCs chronically to animals, but few validation studies exist for reptiles. Two common methods are Silastic® (Dow Corning) implants and cocoa butter injections. Silastic implants containing CORT have been used in several reptile and bird species. For example, subcutaneous implants successfully elevated CORT levels for 5 d posttreatment in Red-eared Slider turtles (*Trachemys scripta elegans*) held in captivity (Cash and Holberton, 1999). Sealed implants have been used intracoelomically to keep plasma CORT levels elevated in Side-blotched Lizards (*Uta stansburiana*), both in short-term laboratory experiments (Denardo and Licht, 1993; Miles et al., 2007) and in long-term (1.5 mo) field studies (Denardo and Sinervo, 1994a,b), although formal validation of the effectiveness of the implants in the long term appears to be lacking. Subcutaneous Silastic implants elevated CORT levels for at least 3 d in Black-legged Kittiwakes (*Rissa tridactyla*), the authors expecting the CORT to be metabolized within 2–3 wk after implantation (Kitaysky et al., 2001), and for 2–4 d in Tree Sparrows (*Spizella arborea*, Astheimer et al., 2000).

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Newman et al. (2010) found that implants with perforations had a higher CORT delivery than did sealed implants in vitro, but still only elevated the baseline levels over those of controls for 2−3 d in vivo in a 28-d validation study in Song Sparrows (*Melospiza melodia*). In birds, Silastic implants with one or both ends open have been used to provide a rapid release of hormone over a limited time period (e.g., Goutte et al., 2010), but sealed or perforated implants are thought to provide a slower release and thus are more likely to be useful if the goal is to elevate CORT chronically.

Because cocoa butter is a lipid that melts at ∼38°C but remains solid at colder temperatures, it has been widely used as a vehicle for corticosteroid injections in fish, mostly in cold water. A single intraperitoneal injection of cortisol-laden cocoa butter can be used to chronically elevate plasma cortisol levels in fish (for a review, see Gamperl et al., 1994). For example, intraperitoneal injections of cortisol in cocoa butter successfully elevated plasma cortisol levels for 5 wk in Brown Trout (*Salmo trutta*) at 12−17°C (Pickering and Duston, 1983). Cortisol-cocoa butter implants were also successfully used in the tropical fish Tilapia (*Oreochromis mossambicus*) at 24−27°C to elevate and maintain cortisol at a level characteristic of a stressed fish for 19 d (Foo and Lam, 1993), which suggests that this method could be successfully used in other ectotherms with similarly warm body temperatures.

In this study, our objective was to assess the effectiveness of two CORT administration methods (Silastic implants and cocoa butter injections) in chronically elevating circulating CORT levels in a temperate reptile, the Painted Turtle (*Chrysemys picta*). To our knowledge, our study is the first to use cocoa butter for CORT treatment in an ectothermic tetrapod. We tested Silastic implants in the field for 2 mo, then in controlled laboratory conditions for 28 d, and cocoa butter injections in the field for 2 mo.

Materials and Methods

Study Species and Study Site.—We used the Painted Turtle (*C. picta*) as our study species because it is the most-abundant local turtle (Juneau, pers. obs.) as well as one of the most-studied and most-widely distributed turtles in North America (Ernst and Lovich, 2009). We captured all turtles in Lake Renaud, a small lake located in Gatineau Park (45°36′10′′N, 76°01′24′′W), Québec, Canada using a dip net, hoop nets (with air space) baited with sardines, or opportunistically by hand from a canoe. At their first capture, we marked turtles semipermanently by notching with a file one to four of their marginal scutes in unique combinations. We released all turtles at their capture location at the end of each experiment.

Experiment 1A: Silastic Implants in the Field.—Silastic implants: In summer 2009, we made slow-release CORT implants using medical grade Silastic tubing (outside diameter = 1.96 mm, inside diameter = 1.47 mm, length = 20 mm; Dow Corning), filled with 20 mg of crystalline CORT (C2505, Sigma Chemicals), and we sealed both ends with Silastic medical adhesive silicone type A (Dow Corning). We soaked the implants in sterile saline prior to implantation. We assigned adult turtles randomly to one of three treatments: two empty Silastic implants (Sham, $n = 10$), one sealed CORT-filled Silastic implant and one empty implant (1 CORT, $n = 9$), or two sealed CORT-filled Silastic implants (2 CORT, $n = 9$). We used a design with two implants in each turtle to test two doses while keeping constant the number and length of the implants. After applying a local topical anesthetic (cream lidocaine) on the skin, we inserted the implants subcutaneously, one in each thigh, using a syringe-style implanter with a 12-ga needle (MK10 implanter with N125 needle, Biomark, ID, USA). We sealed the puncture site with surgical glue and allowed the glue to dry thoroughly (up to 20 min) before releasing individuals at their capture location. Over 2 mo (67 d), we opportunistically recaptured and blood-sampled 28 implanted turtles in the field.

Blood sampling: On day 0 (before the implantation) and over a 2-mo period during the summer, we collected baseline blood samples to determine whether the implants elevated circulating baseline CORT levels over time. We used the moment at which the turtle first noticed the presence of the capturer as the starting time (t = 0 min). Immediately after capture, and no later than t = 10 min (Cash et al., 1997) (mean = 4.20 min, SD = 2.25 min, $n = 94$), we collected a first blood sample (300−500 μL) from the coccygeal vein (Bulté et al., 2006) using preheparinized syringes (0.5 cc with a 28-ga needle). We stored blood samples in an ice slurry for up to 8 h and then we centrifuged them to collect plasma. We snap-froze plasma samples on dry ice and then placed them in a freezer at −80°C. Using samples collected within 10 min on day 0, we observed no significant relationship between bleed time and baseline CORT level (linear regression, $R^2 = 0.075$, $P = 0.16$, $n = 28$) and found no difference between baseline CORT levels from samples collected within and after 5 min (*t*-test; $t_{26} = 0.615$, $P = 0.54$). On day 0, baseline CORT levels were not different between the sexes (*t*-test; $t_{26} = 0.50$, $P = 0.62$, $n = 5$ females and 23 males); therefore data from males and females were combined in the analyses. On day 0, baseline CORT levels were not different among treatment groups (one-way analysis of variance [ANOVA]; $F_{2,25} = 0.69$, $P = 0.51$). Following implantation (day 0), turtles were recaptured 1–6 times (mean = 2.1, median = 2) over 2 mo (67 d, Fig. 1).

Experiment 1B: Silastic Implants in the Laboratory.—Animal collection and husbandry: In August 2010, we captured 28 adult male Painted Turtles (as described above). We used only adult males to avoid any potential confounding effect of developmental or reproductive stage. We measured and weighed turtles and drove them back the same day to the University of Ottawa. Before starting the experiment, we allowed turtles to acclimate to captivity for 12.8 d on average (±1.1 d, no difference among treatment groups, $P = 0.99$). We housed turtles individually in vivaria containing water and a basking platform, kept them at the preferred temperature for the species (∼23°C, typical in the field, Edwards and Blouin-Demers, 2007) on a natural photoperiod cycle (13 L: 11 D), fed them with earthworms twice a week, and provided them with fresh, dark leafy greens ad libitum.

Silastic implants: We made and inserted slow-release CORT implants as described for Experiment 1A, with the exception that each turtle received only one implant instead of two. We assigned turtles randomly to one of four treatments ($n = 7$ in each group): no implant (Control), one empty Silastic implant (Sham), one sealed CORT-filled Silastic implant (CORT sealed), or one CORT-filled Silastic implant perforated with 8 holes (made with a 25-ga needle) to increase delivery (CORT holes). We sealed the puncture site with surgical glue and allowed the glue to dry thoroughly (up to 20 min) before placing individuals back into vivaria. At the end of the study, we made a small incision with a scalpel through anesthetized skin to remove the implants and then sealed the skin with surgical glue.

Blood sampling and animal processing: Prior to implantation on day 0, then on days 2, 4, 7, 14, 21, and 28, we collected baseline blood samples as described for Experiment 1A (mean =

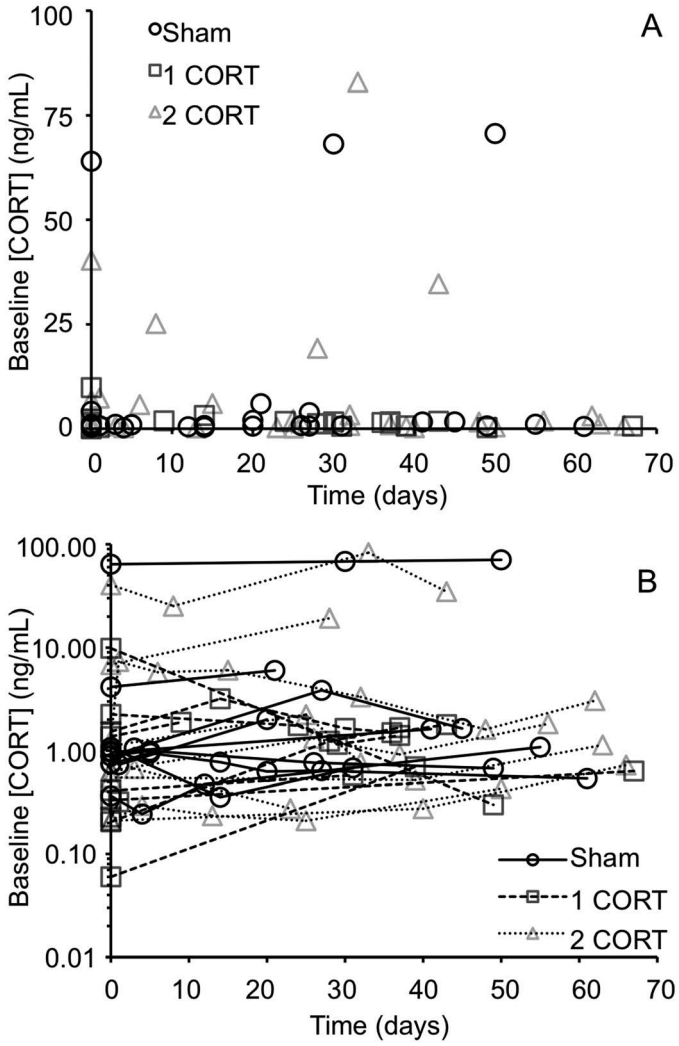

FIG. 1. Plasma total baseline corticosterone concentrations ([CORT]) as a function of time since treatment in free-living adult Painted Turtles (*Chrysemys picta*) (90 data points from 28 individuals) in Experiment 1A. Individuals were randomly assigned to one of three treatment groups: two empty Silastic implants (Sham, $n = 10$), one sealed CORT-filled Silastic implant and one empty implant (1 CORT, $n = 9$), or two sealed CORT-filled Silastic implants (2 CORT, $n = 9$). (A) [CORT] values are presented on a linear scale. (B) Individual profiles over time. [CORT] values are presented on a logarithmic scale to allow better visualization of each profile.

4.17 min, SD = 2.95 min, $n = 196$) to determine whether the implants elevated the circulating baseline CORT levels over time. Using samples collected within 10 min on day 0, we observed no relationship between bleed time and baseline CORT level (linear regression, $R^2 = 0.009$, $P = 0.65$, $n = 26$) and found no difference between baseline CORT levels from samples collected within and after 5 min (*t*-test; $t_{22.1} = -0.412$, $P = 0.68$). On days 0, 7, and 28 we collected a stress-induced sample after 30 min (mean = 35.45 min, SD = 3.55 min, $n = 84$) of restraint in a plastic bin to determine whether CORT treatment reduced stress responsiveness. On day 0, baseline CORT levels were not different among treatment groups (one-way ANOVA; $F_{3,24} = 1.78$, $P = 0.18$, $n = 28$, Fig. 2A) nor were stress-induced levels (one-way Welch ANOVA; $F_{3,12.98} = 0.37$, $P = 0.77$, $n = 28$, Fig. 2A). On each sampling day, we weighed the turtles.

Experiment 2: Cocoa Butter Injections.—Cocoa butter implants: In summer 2011, we dissolved crystalline CORT (C2505, Sigma Chemicals) in liquid pure cocoa butter (10 mg CORT/1 mL butter). We kept this mixture in a warm water bath and injected it epicoelomically (near the pectoral muscle) to CORT-treated individuals ($n = 17$) using a 1-mL syringe and 18-ga, 38-mm-long needles at a dose of 10 mg CORT in 1 mL cocoa butter per 1 kg body mass, then we sealed the injection site with surgical glue. Sham-treated individuals ($n = 5$) received an injection of cocoa butter only while controls ($n = 6$) received no injection. Over 2 mo, we opportunistically recaptured and blood-sampled 28 adult turtles in the field.

Blood sampling: On day 0 (before the cocoa butter injection) and over a 60-d period during the summer, we collected baseline blood samples as described for Experiment 1A (mean = 4.89 min, SD = 2.42 min, $n = 22$) to determine whether the implants elevated circulating baseline CORT levels over time. Using samples collected within 10 min on day 0, we observed no relationship between bleed time and baseline CORT level (linear regression, $R^2 = 0.0002$, $P = 0.95$, $n = 22$) and found no difference between baseline CORT levels from samples collected within and after 5 min (*t*-test; $t_{15.2} = 0.359$, $P = 0.72$). On day 0, we also collected a stress-induced sample as described for Experiment 1B (mean = 33.91 min, SD = 1.72 min, $n = 22$). On day 0, baseline CORT levels were not different between the sexes (*t*-test; $t_{20} = 0.60$, $P = 0.55$, $n = 5$ females and 17 males), nor were stress-induced levels (*t*-test; $t_{20} = 0.38$, $P = 0.71$), and therefore data from males and females were combined in the analyses. On day 0, baseline CORT levels were not different among treatment groups (one-way ANOVA; $F_{2,19} = 0.75$, $P = 0.49$, $n = 4$ control, 5 sham, 13 CORT, Fig. 3A) nor were stress-induced levels (one-way ANOVA; $F_{2,19} = 0.01$, $P = 0.99$, Fig. 3A). Following injection (day 0), turtles were recaptured 1–5 times (mean = 2, median = 2) over 60 d.

Corticosterone Assays.—We determined total CORT concentrations using a competitive enzyme-linked immunosorbent assay (ELISA) (sensitivity 27 pg/mL, Cat. No. 900-097, Assay Designs Inc.). We used the kit as per the manufacturer's instructions. We diluted our samples 5 to 50 times in the provided assay buffer to obtain concentrations within the range of the standard curve. To a known volume of plasma, we added the same volume of the provided steroid displacement reagent (previously diluted 1 : 100 in assay buffer), let sit 10 min, then added assay buffer to reach the desired dilution. We used SoftMax Pro 4.0 (Molecular Devices) to calculate the concentration of CORT in the samples from the optical density data. Using serial dilutions of turtle plasma samples, we obtained curves that were parallel to standard curves.

We ran all standard curves in triplicate (mean coefficient of variation [CV] = 3.3%) and all samples in duplicate (mean CV = 11.2%). We determined an intra-assay coefficient of variation of 10.9% by running a randomly chosen sample in two sets of duplicates on a given plate, and an interassay variation of 30.0% by running duplicated aliquots from a pool of samples on each plate, and of 20.4% by running duplicates of the same samples on different plates. We kept all samples from a given individual on the same plate, and we had individuals from each treatment group on each plate we ran. This sample randomization mitigated the potential effect of the high interassay variation on our data.

Statistical Analyses.—We conducted all statistical analyses using the statistical software JMP (versions 5 and 8, SAS). We log-transformed data as needed to satisfy assumptions of normality

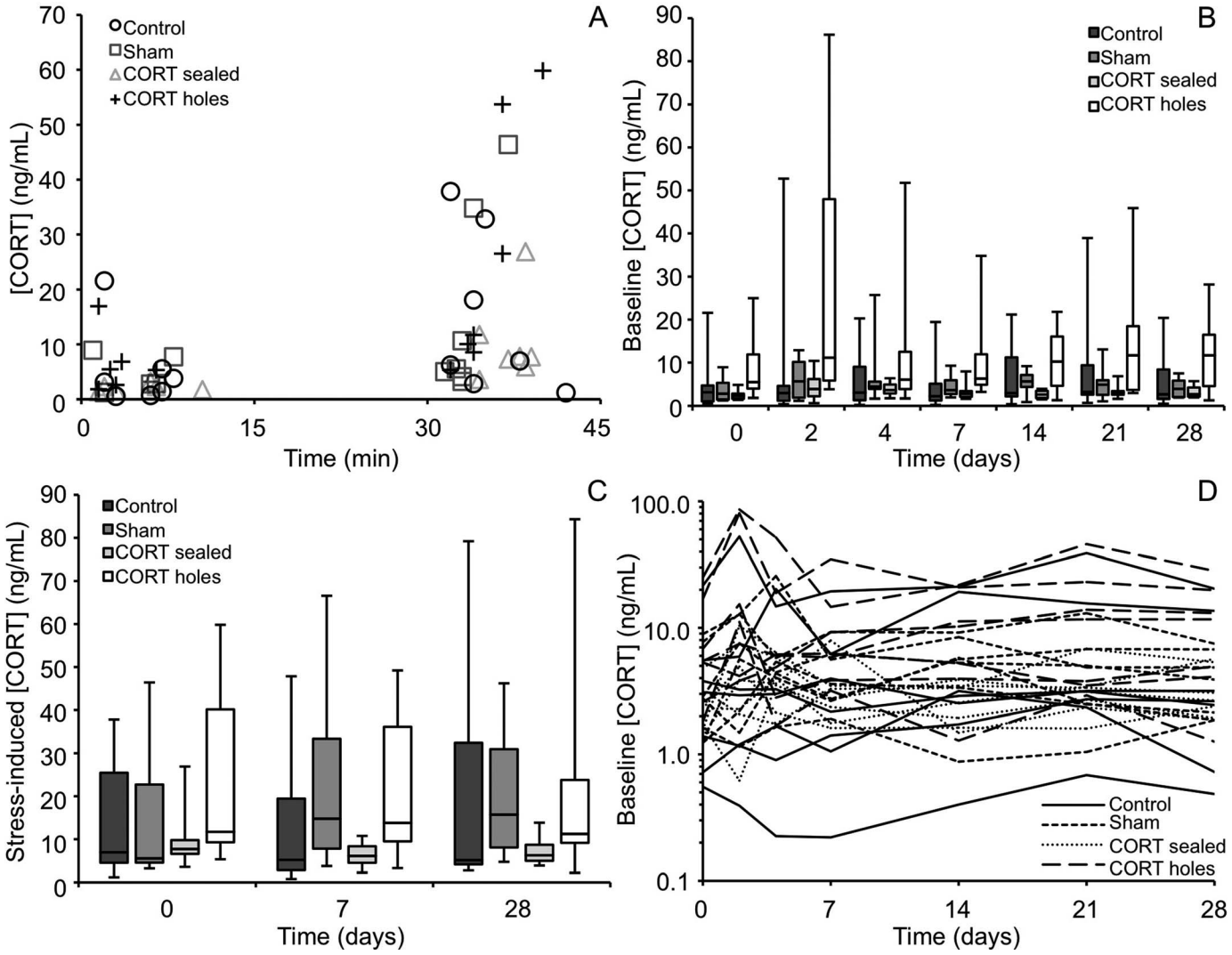

FIG. 2. Plasma total corticosterone concentrations ([CORT]) from captive adult male Painted Turtles (*C. picta*) in Experiment 1B. Individuals were randomly assigned to one of four treatment groups ($n = 7$ for each group): no implant (Control), one empty Silastic implant (Sham), one sealed CORT-filled Silastic implant (CORT sealed), or one perforated CORT-filled Silastic implant (CORT holes). Baseline samples were collected as quickly as possible after taking the turtle out of its tank while stress-induced samples were collected following 30 min of restraint. (A) Baseline (scatter on left) and stress-induced (scatter on right) [CORT] on day 0, prior to treatment, as a function of time since first handling the animal ($n = 28$ pairs). (B) Baseline [CORT] as a function of time since implantation. (C) Stress-induced [CORT] as a function of time since implantation. (D) Individual profiles of baseline [CORT] over time. [CORT] values are presented on a logarithmic scale to allow better visualization of each profile.

(tested with Shapiro-Wilk) and homogeneity of variances (tested with Levene's test).

Experiment 1A: Silastic implants in the field: To assess whether CORT treatment elevated baseline CORT levels, we used an analysis of covariance (ANCOVA) of baseline CORT levels (excluding day 0) vs. treatment group and time to determine whether the slopes differed among the groups.

Experiment 1B: Silastic implants in the laboratory: We used a paired *t*-test to determine whether baseline and stress-induced levels differed among individuals on day 0. To determine whether CORT treatment affected baseline CORT levels and stress responsiveness, we analyzed baseline and stress-induced CORT levels vs. time and treatment group using two-way ANOVAs with repeated measures. To determine whether treatment affected body condition, we used a *t*-test to ascertain whether the mass lost during the experiment (% difference between days 0 and 28) differed from 0 and a one-way ANOVA to find whether this mass loss differed among groups.

Experiment 2: Cocoa butter injections: We used a paired *t*-test to determine whether baseline and stress-induced levels differed among individuals on day 0. To assess whether CORT treatment elevated baseline CORT levels, we used an ANCOVA of baseline CORT levels (excluding day 0) vs. treatment group and time to determine whether the slopes differed among the groups, then performed linear regressions of baseline CORT levels (excluding day 0) vs. time for each treatment group.

RESULTS AND DISCUSSION

Experiment 1A: Silastic Implants in the Field.—During the experiment, the Painted Turtles exhibited baseline CORT levels (Fig. 1A; Table 1) similar to those previously observed in other Painted Turtles (Keiver et al., 1992) and Red-eared Sliders (Cash et al., 1997). On day 0, baseline CORT levels for all groups combined ($n = 28$) were: mean = 5.1 ng/mL; SE = 2.6; median = 0.9; min = 0.1; max = 64.0. In Table 1, we report mean (±SE) and

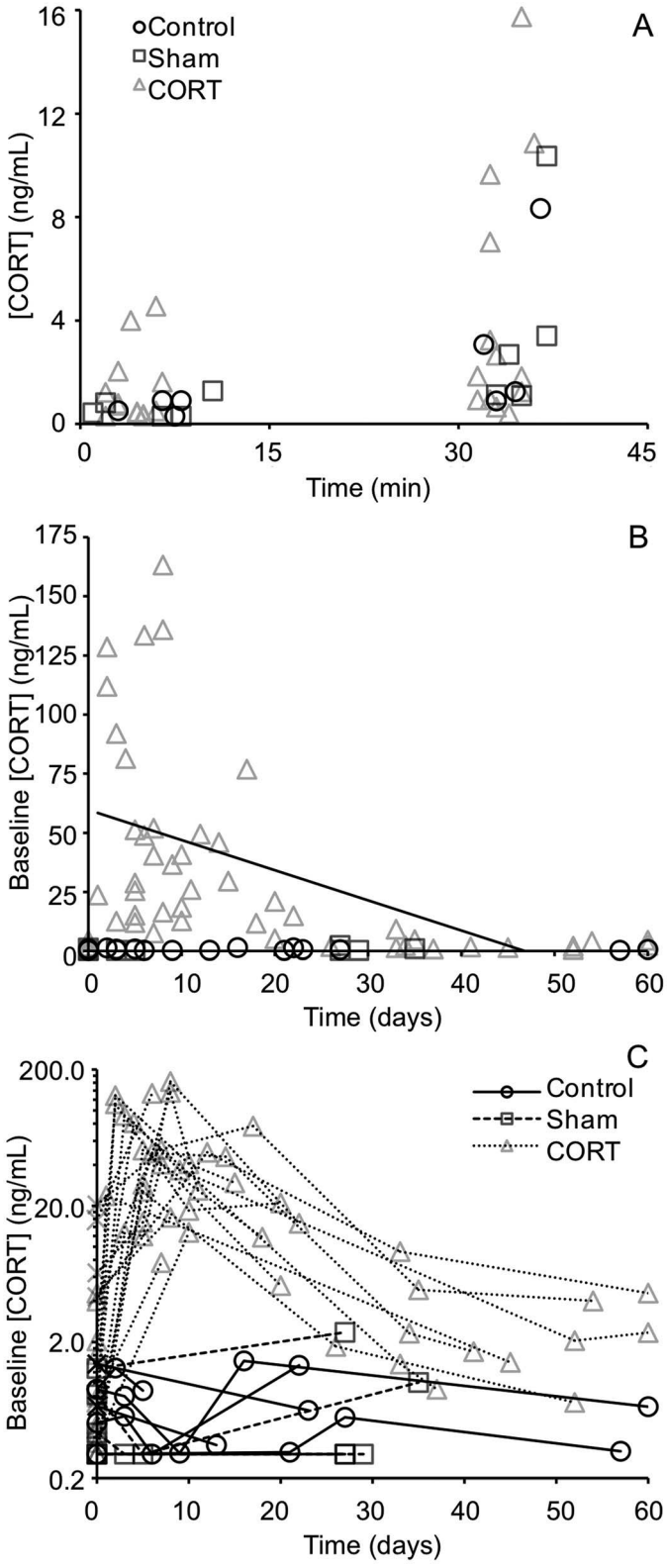

FIG. 3. Plasma total corticosterone concentrations ([CORT]) from free-living adult Painted Turtles (*C. picta*) in Experiment 2. Individuals were randomly assigned to one of three treatment groups: without injection (Control), injected with cocoa butter only (Sham), or injected with CORT-laden cocoa butter (CORT). Baseline samples were collected as quickly as possible after disturbing the turtle while stress-induced samples were collected following a 30-min restraint. (A) Baseline (scatter on left) and stress-induced (scatter on right) [CORT] on day 0, prior to treatment, as a function of time since first handling the animal ($n = 22$ pairs). (B) Baseline [CORT] as a function of time since treatment (88 data points from 28 individuals: 6 controls, 5 sham-treated, and 17 CORT-treated). The solid line shows the significant linear regression calculated after day 0 for the CORT-treated group. Regressions were not significant for the other groups. (C) Individual profiles of baseline [CORT] over time. [CORT] values are presented on a logarithmic scale to allow better visualization of each profile. Six baseline samples ($n = 2$ Control and 4 CORT) collected after 10 min (11–21 min) on day 0 were excluded from other analyses and figures but are shown (×) to allow presentation of all profiles and still demonstrate increases because of CORT treatment.

median baseline CORT concentrations on day 0 and for various time periods for each treatment group. It is worth noting that two individuals had higher baseline CORT concentrations starting on day 0 (Fig. 1) and which remained high throughout the experiment. The elevated means at certain time points were mostly driven by these individuals and were not related to treatment. Therefore, mean values should be interpreted with caution, and median values provide a more-accurate representation of central tendency. Over the 2 mo of the experiment, baseline CORT levels remained low for all groups (Fig. 1A; Table 1). Individual profiles (Fig. 1B) indicate that CORT values were fairly constant through time for most individuals, with some variability that did not appear to be associated with treatment and high variability among individuals. For baseline CORT levels measured on or after day 1 ($n = 62$ data points from 28 individuals: 25 from 9 individuals with 2 CORT implants, 15 from 9 individuals with 1 CORT implant, and 22 from 10 individuals with sham implants), we found a nonsignificant group*time interaction ($P = 0.34$), a nonsignificant effect of group ($P = 0.57$), and a nonsignificant effect of time ($P = 0.81$). This nonsignificant interaction term means that the slopes of the relationship did not differ for the three groups. The results suggest that the sealed Silastic implants do not elevate circulating levels of CORT over baseline levels in free-living Painted Turtles.

Experiment 1B: Silastic Implants in the Laboratory.—On day 0 of the experiment, the male Painted Turtles had baseline CORT levels (Fig. 2A) (mean = 5.2 ng/mL; SE = 1.2; median = 2.8; min = 0.6; max = 25.0; $n = 28$ for all groups combined) similar to those previously observed in other Painted Turtles (Keiver et al., 1992) and Red-eared Sliders (Cash et al., 1997). Turtles exhibited the typical vertebrate stress response, with stress-induced CORT levels being higher than baseline levels after 30 min of handling and restraint ($t_{27} = 9.65$, $P < 0.001$, $n = 28$, Fig. 2A) (mean = 16.5 ng/mL; SE = 3.1; median = 8.2; min = 1.2; max = 59.8; $n = 28$ for all groups combined). Three individuals (one Control and two CORT holes) had unusually high baseline CORT levels on day 0 (Fig. 2A) and consistently higher levels throughout the experiment. The presence of these three individuals in the analyses does not qualitatively change the conclusions, and there is no particular reason to exclude them; therefore, we left them in the data set. We report mean (±SE) and median baseline (Table 2) and stress-induced (Table 3) CORT concentrations on day 0 and for various time points for each treatment group. The elevated means at certain time points were driven by these three individuals and were not related to treatment. Therefore, mean values should be interpreted with caution, and median values (Fig. 2B,C) provide a more-accurate representation of central tendency. Individual profiles (Fig. 2D) indicate that baseline CORT values were somewhat variable within individuals, with some variability that did not appear to be associated with treatment and high variability among individuals. Some profiles from each treatment group (including Control and Sham) show a rise at day 2 or 4 (or both) while CORT levels remained steady or

TABLE 1. Plasma total baseline corticosterone concentrations ([CORT]) for various time intervals after treatment in free-living adult Painted Turtles (*C. picta*) in Experiment 1A. Median, mean, standard error (SE), and sample size (*n*) are presented. Individuals were randomly assigned to one of three treatment groups: two empty Silastic implants (Sham), one sealed CORT-filled Silastic implant and one empty implant (1 CORT), or two sealed CORT-filled Silastic implants (2 CORT). Only the first sample was included for individuals that were sampled twice within the same time interval ($n = 2$).

		Baseline [CORT] (ng/mL)			
Treatment group	Days postimplant	Median	Mean	SE	*n*
Sham	0	0.9	7.5	6.3	10
	1–4	0.7	0.7	0.2	3
	5–10	1.0	1.0	0.1	2
	11–20	0.6	0.9	0.4	4
	21–30	3.9	15.9	13.1	5
	31–40	0.7	0.7	—	1
	41–50	1.6	18.7	17.3	4
	51–67	0.8	0.8	0.3	2
1 CORT	0	0.4	1.8	1.0	9
	1–4	0.3	0.3	—	1
	5–10	1.9	1.9	—	1
	11–20	3.2	3.2	—	1
	21–30	1.7	1.5	0.2	3
	31–40	1.4	1.2	0.2	5
	41–50	1.1	1.1	0.8	2
	51–67	0.6	0.6	—	1
2 CORT	0	0.6	5.8	4.4	9
	1–4	0.7	2.8	2.3	3
	5–10	15.5	15.5	9.7	2
	11–20	3.1	3.1	2.9	2
	21–30	1.3	4.7	3.7	5
	31–40	0.8	14.8	13.6	6
	41–50	1.6	12.3	11.2	3
	51–67	1.5	1.7	0.5	4

declined in others. Overall, we did not observe a clear effect of treatment group on baseline CORT levels over the duration of the experiment (Fig. 2B, D). We detected no time*group interaction ($F_{18,54.2} = 0.99$, $P = 0.49$), and no effect of treatment group ($F_{3,24} = 2.10$, $P = 0.13$), but did detect a significant effect of time ($F_{6,19} = 3.39$, $P = 0.02$). This time effect likely resulted from captivity stress. These results indicate that the Silastic implants of CORT did not elevate circulating baseline CORT levels on days 2–28. For stress-induced levels (Fig. 2C), we found no time*group interaction ($F_{6,46} = 1.63$, $P = 0.16$), no effect of treatment group ($F_{3,24} = 1.10$, $P = 0.37$), and no effect of time ($F_{2,23} = 0.67$, $P = 0.52$). Therefore, the Silastic CORT treatment did not affect stress responsiveness in these turtles. Turtles lost an average of 1.45 ± 0.48% of body mass over the 28 d of the experiment (significantly different from 0, $t_{27} = 3.06$, $P = 0.005$); however, mass loss was the same across the treatment groups ($F_{3,24} = 0.72$, $P = 0.55$).

A visual inspection of the implants after their removal from the animals suggested that the implants with holes released some CORT during the experiment whereas the sealed implants did not appear to release any CORT. A possible explanation for the absence of a measureable increase in circulating CORT levels in the animals with perforated implants is that this CORT was excreted rapidly, preventing any detectable elevation in circulating CORT levels as well as any effects on the acute stress response and body condition. The renal portal system, present in most nonmammalian vertebrates including turtles, and thought to collect blood from the lower body and bring a portion of this blood to the kidneys, could potentially lead to a rapid excretion of drugs injected into the lower body and thus affect their efficacy. Holz et al. (1997a) tested this possibility in Red-eared Slider Turtles and concluded that injection site was unlikely to cause clinically significant effects on renal extraction of drugs through the renal portal system and that the lower

TABLE 2. Plasma total baseline corticosterone concentrations ([CORT]) at various time points after treatment in captive adult male Painted Turtles (*C. picta*) in Experiment 1B. Median, mean, and standard error (SE) are presented. Individuals were randomly assigned to one of four treatment groups ($n = 7$ in each group): no implant (Control), one empty Silastic implant (Sham), one sealed CORT-filled Silastic implant (CORT sealed), or one perforated CORT-filled Silastic implant (CORT holes).

		Baseline [CORT] (ng/mL)		
Treatment group	Days postimplant	Median	Mean	SE
Control	0	3.1	5.2	2.8
	2	2.9	9.7	7.2
	4	3.0	6.3	3.0
	7	2.2	4.9	2.5
	14	2.9	7.3	3.4
	21	3.1	9.5	5.3
	28	2.6	6.2	2.9
Sham	0	2.8	3.8	1.2
	2	5.7	6.2	1.9
	4	4.4	7.3	3.1
	7	3.6	4.6	1.0
	14	5.6	5.5	1.1
	21	4.9	5.1	1.5
	28	3.9	4.1	0.9
CORT sealed	0	2.3	2.5	0.5
	2	3.9	4.5	1.3
	4	3.6	3.9	0.7
	7	2.8	3.3	0.8
	14	2.6	2.6	0.4
	21	3.1	3.3	0.6
	28	2.7	3.3	0.6
CORT holes	0	5.5	9.1	3.3
	2	11.1	29.8	13.9
	4	6.1	13.2	6.8
	7	6.3	11.1	4.2
	14	10.2	10.7	3.1
	21	11.7	15.0	5.8
	28	11.7	11.9	3.6

TABLE 3. Plasma total stress-induced corticosterone concentrations ([CORT]) at various time points after treatment in captive adult male Painted Turtles (*C. picta*) in Experiment 1B. Median, mean, and standard error (SE) are presented. Individuals were randomly assigned to one of four treatment groups ($n = 7$ in each group): no implant (Control), one empty Silastic implant (Sham), one sealed CORT-filled Silastic implant (CORT sealed), or one perforated CORT-filled Silastic implant (CORT holes).

		Stress-induced [CORT] (ng/mL)		
Treatment group	Days postimplant	Median	Mean	SE
Control	0	7.0	15.1	5.6
	7	5.2	14.1	6.9
	28	5.1	22.9	11.0
Sham	0	5.5	15.7	6.6
	7	14.8	23.9	9.3
	28	15.7	20.7	6.5
CORT sealed	0	7.7	10.1	3.0
	7	6.1	6.4	1.1
	28	6.3	7.3	1.3
CORT holes	0	11.7	25.1	8.6
	7	13.8	22.5	6.9
	28	11.2	23.4	10.8

TABLE 4. Plasma total baseline corticosterone concentrations ([CORT]) for various time intervals after treatment in free-living adult Painted Turtles (*C. picta*) in Experiment 2. Median, mean, standard error (SE), and sample size (*n*) are presented. Individuals were randomly assigned to one of three treatment groups: without injection (Control), injected with cocoa butter only (Sham), or injected with CORT-laden cocoa butter (CORT). Only the first sample was included for individuals that were sampled twice within the same time interval ($n = 6$).

		Baseline [CORT] (ng/mL)			
Treatment group	Days postimplant	Median	Mean	SE	*n*
Control	0	0.7	0.7	0.2	4
	1–4	0.8	0.9	0.2	3
	5–10	0.3	0.5	0.2	3
	11–20	0.9	0.9	0.6	2
	21–30	0.6	0.8	0.3	3
	31–40	—	—	—	0
	41–50	—	—	—	0
	51–60	0.5	0.5	0.2	2
Sham	0	0.4	0.6	0.2	5
	1–4	0.3	0.3	—	1
	5–10	0.3	0.3	—	1
	11–20	—	—	—	0
	21–30	0.3	0.8	0.5	4
	31–40	1.0	1.0	—	1
	41–50	—	—	—	0
	51–60	—	—	—	0
CORT	0	0.7	1.3	0.4	13
	1–4	86.7	75.0	19.2	6
	5–10	27.2	35.7	8.6	14
	11–20	27.8	33.2	8.2	8
	21–30	8.4	8.4	6.5	2
	31–40	3.6	4.4	1.8	4
	41–50	1.6	1.6	0.1	2
	51–60	3.0	2.8	0.9	4

body could be used for drug administration in reptiles. In a companion study (Holz et al., 1997b), however, they demonstrated that circulation from the hindlimbs flowed mainly to the liver and bypassed the kidneys, contrary to what was previously thought. Because GCs are metabolized by target cells or the liver (Norris, 2007), it is possible that part of the CORT we administered was metabolized after a first pass in the liver, reducing its concentration in the blood and its effect on other tissues. Some researchers also leave one end of the Silastic implants open to improve CORT delivery, but this is typically used for short-term rapid release, not for a chronic elevation of CORT. It appears that sealed and perforated Silastic implants cannot be used to chronically elevate circulating CORT levels in Painted Turtles.

Experiment 2: Cocoa Butter Injections.—On day 0 of the experiment, the Painted Turtles had baseline CORT levels (mean = 1.1 ng/mL; SE = 0.2; median = 0.6; min = 0.3; max = 4.6; $n = 22$ for all groups combined) (Fig. 3A) similar to those previously observed in other Painted Turtles (Keiver et al., 1992) and Red-eared Sliders (Cash et al., 1997) and overlapping with the lower values measured in captive male turtles in Experiment 1B (Fig. 2A). Turtles in this field-based experiment also exhibited the typical vertebrate stress response, with stress-induced CORT levels being higher than baseline levels after 30 min of handling and restraint ($t_{21} = 6.82$, $P < 0.001$, Fig. 3A) (mean = 4.1 ng/mL; SE = 0.9; median = 2.3; min = 0.4; max = 15.7; $n = 22$ for all groups combined). In Table 4, we report mean (±SE) and median baseline CORT concentrations on day 0 and for various time periods for each treatment group.

Over the 2 mo of the experiment, baseline CORT levels remained low for control and sham-treated turtles while they were elevated starting on day 1 and for up to 3 wk for CORT-treated individuals (Fig. 3B). In individual profiles (Fig. 3C) of control and sham-treated turtles, CORT levels remained steady or slightly decreased within the first week and then showed some variability not associated with treatment and within the baseline range. In CORT-treated individuals, however, there was a rapid or progressive increase in CORT levels over the first 7–10 d followed by a slower decline around the third week. Despite large interindividual variation, this observation suggests that the injection of CORT in cocoa butter can be used to elevate circulating levels of CORT over baseline levels in free-living Painted Turtles for a period of a few weeks. For baseline CORT levels measured on or after day 1 ($n = 66$ data points from 28 individuals: 14 from 6 controls, 7 from 5 shams, and 45 from 17 CORT-treated individuals), we found a significant group*time interaction ($P < 0.001$), a significant effect of group ($P < 0.001$), and a nonsignificant effect of time ($P = 0.27$). This significant interaction term means that, as expected, the slopes of the relationship differed for the three groups. To make sure this effect was not because of pseudoreplication, we repeated the analysis using only one randomly selected point for each individual ($n = 6$ controls, 5 shams, and 17 CORT-treated individuals), and we found a marginally significant group*time interaction ($P = 0.09$), a significant effect of group ($P < 0.001$), and a nonsignificant effect of time ($P = 0.32$). As expected, we found a significant negative linear relationship of baseline CORT levels over time for the CORT-treated group ($R^2 = 0.58$, $F_{1,43} = 60.52$, $P < 0.001$, $n = 45$) but not for the control ($R^2 = 0.02$, $F_{1,12} = 0.19$, $P = 0.67$, $n = 14$) or the sham-treated group ($R^2 = 0.18$, $F_{1,5} = 1.09$, $P = 0.35$, $n = 7$).

To the best of our knowledge, there are no published data on patterns of CORT concentrations in wild Painted Turtles that experience natural stressors. Therefore, we need to rely on experimental studies to assess whether our achieved concentrations were relevant in an ecological and physiological context (as opposed to delivering pharmacological doses). Although it does not appear that the pattern is ubiquitous (Dickens and Romero, 2013), in some situations chronic stress can result in baseline CORT levels elevated into the range of values typical of acute stress (Bonier et al., 2009) for the duration of the chronic stress. For example, Keiver et al. (1992) reported that cannulated Painted Turtles undergoing 10 h of underwater anoxia at 22°C in laboratory conditions had CORT levels in the typical baseline range (0.8 to 4.5 ng/mL in their study) while catecholamines increased greatly. However, when anoxic turtles were allowed to surface and recover, catecholamines rapidly decreased while CORT levels increased 10-fold over controls after 4 h of recovery (mean = 19.0 ng/mL, SD = 9.4, $n = 4$), were still elevated after 10 h (mean = 15.5 ng/mL, SD = 2.5, $n = 4$), and slowly decreased until 48 h of recovery, suggesting a role for glucocorticoids in the recovery from anoxic stress. Larocque et al. (2012a,b) demonstrated that freshwater commercial fisheries result in bycatch of Painted Turtles, and that turtles in such submerged nets experience anoxia, which can lead to substantial turtle mortality (up to 33%). Therefore, it is likely that turtles recovering from entrapment in fishing nets would be secreting higher CORT levels.

To ascertain to what extent Painted Turtles caught in commercial hoop net fisheries experience such CORT secretion during recovery, we submitted turtles ($n = 9$) to 7 h of anoxia in submerged hoop nets in Lake Opinicon (eastern Ontario,

Canada) (following methods of Larocque et al., 2012b) after which we took turtles out of the nets, allowed them to recover on a platform, and blood-sampled them via venipuncture. When removed from the nets, turtles had typical baseline CORT levels (mean = 3.8 ng/mL, SD = 4.7) which then became elevated after 1 h (mean = 9.6 ng/mL, SD = 12.8, max = 41.9) and 4 h (mean = 9.4 ng/mL, SD = 7.9, max = 23.4) of recovery (Juneau and Larocque, pers. obs.). Although some of our cocoa butter CORT-treated individuals experienced higher levels than those of entrapped turtles, mostly in the first week of treatment (Fig. 3B), a majority (57%) of samples collected from CORT-treated turtles between days 1 and 21 were below the maximum that we measured after 1 h of recovery from anoxia and 33% below the maximum after 4 h. These results, combined with those of Keiver et al. (1992), indicate that most of our cocoa butter CORT-treated turtles experienced CORT levels in the same range as turtles recovering from entrapment in fishing nets, which is probably a relevant stressor for a natural population of freshwater turtles, although our treated turtles may have experienced elevated CORT for longer than would wild animals. Moreover, the baseline CORT levels we achieved with our cocoa butter dosage are similar to stress-induced levels measured in free-living Painted Turtles captured for the first time in Lake Renaud in the summer of 2009 and submitted to a 30-min standardized stress-restraint protocol (mean = 8.5 ng/mL, SD = 17.6, max = 91.4, n = 49; Juneau, pers. obs.). Taken together, these data suggest that the achieved CORT concentrations from our cocoa butter injections were probably physiologically and ecologically relevant for the species (and not pharmacological doses). Chronic stress can result in baseline CORT levels elevated in the range of acute stress (Bonier et al., 2009) for the duration of the chronic stress, at least in some situations. Thus, cocoa butter injections can be used to chronically elevate CORT levels to likely ecologically relevant levels.

Conclusions

We tested two methods to chronically elevate CORT levels in a temperate reptile, the Painted Turtle (*C. picta*). In the first experiment, we used sealed Silastic implants of CORT in free-living turtles and observed no effect on baseline CORT levels. We then used sealed and perforated Silastic implants in captive turtles and observed no significant difference in baseline CORT levels, hormonal stress response, or body mass among treatment groups. Our findings suggest that sealed or perforated Silastic implants of CORT do not necessarily function as previously expected and should be used with caution. In the second experiment, we investigated the usefulness of injections of CORT-laden cocoa butter for the first time in an ectothermic tetrapod. Despite large interindividual variation, we found that generally, circulating CORT levels were elevated for up to 3 wk in free-living turtles at concentrations that were likely physiologically and ecologically relevant for the species, i.e., that were mostly around typical stress-induced levels or at levels experienced by turtles recovering from entrapment in commercial fishing nets. The duration of the CORT elevation, however, possibly was longer than that experienced by wild turtles. We conclude that cocoa butter injections, but not sealed or perforated Silastic implants, of CORT can be used in Painted Turtles to chronically elevate circulating CORT levels and represent a promising method for other ectotherms, such as amphibians and other reptiles, living in temperate climates.

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